# ANALYSIS OF GENE EXPRESSION IN EDNRA MUTANT EMBRYOS

A Thesis

by

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# ABSTRACT

Endothelin receptor A (Ednra) signaling pathway is involved in many pathological and physiological conditions, yet the pathway and the genes regulated by the pathway remain largely unknown. *Ednra* mutant murine embryos are known to present with cardiovascular defects including patent ductus arteriosus, coarctation of the aortic arch, ventricular septal defects indicating issues with remodeling of the pharyngeal arch arteries, and improper development of the cardiac outflow tract among others. To elucidate the expression of specific genes involved during cardiovascular development in these mutants we first generated *Ednra-/-* murine embryos and performed whole mount *in situ* hybridization at embryonic day E9.5 and discovered altered gene activity that could explain the cardiovascular defects seen in these mutants. We also looked into the problem of cardiac neural crest cell migration using immunohistochemistry to analyze the Rho family of GTPases. We discovered that in the cardiac outflow tract neural crest cells, Endothelin receptor A signaling involves Cdc42 activity.

# DEDICATION

To my loved ones for giving me the encouragement and support and to all those in the pursuit of education: never give up.

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# NOMENCLATURE

| | |
|---|---|
| ANP | Atrial natriuretic peptide |
| BMP | Bone morphogenic protein |
| CCR1 | Chemokine Receptor 1 |
| CNCC | Cardiac neural crest cells |
| COFT | Cardiac outflow tract |
| E | Embryonic day |
| ECE1 | Endothelin Converting Enzyme 1 |
| ECE2 | Endothelin Converting Enzyme 2 |
| Edn1 | Endothelin 1 |
| Ednra | Endothelin A receptor, Endothelin receptor A |
| FGF | Fibroblast growth factor |
| NCC | Neural crest cells |
| Nppa | Natriuiretic peptide A |
| PAA | Pharyngeal arch arteries |
| PCR | Polymerase chain reaction |

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# 1. INTRODUCTION AND LITERATURE REVIEW

Congenital cardiovascular defects affect one in 110 births; such a high prevalence of defects in humans is a driving force in understanding the genes and pathways involved during normal heart development. The heart is the first functional organ to form in a developing embryo. Cardiovascular development is a long and complex process beginning before the neural tube is formed and finishing after birth with the closure of the ductus arteriosus (Carlson 2013). The development of the heart begins with the formation of two endocardial tubes within the anterior mesoderm that merge to form the primitive heart tube (Carlson 2013). Rapid looping and differentiation of the tubular heart into the truncus arteriosus, bulbus cordis, primitive ventricle, primitive atrium and sinus venosus occurs in early development (Carlson 2013). The truncus arteriosus will divide and form the pulmonary artery and aorta; the bulbus cordis will develop into the right ventricle; the primitive ventricle will form the left ventricle; the primitive atrium forms the anterior portion of the left and right atrium; the sinus venosus contributes to the posterior part of the right atrium (Carlson 2013). The basic cellular unit of the heart is the cardiomyocyte, with unique subtypes categorized by location and specialized function. While much is known about the myocardium of the heart, there is relatively little understood about the factors involved in maturation of these cell lineages.

One cell population involved in the development of the cardiovascular region is a group known as the neural crest cells. Neural crest cells are multipotent cells that delaminate from the ectoderm layer following the transition from epithelial to

mesenchymal cell types during early embryogenesis. A subpopulation of these cells become what is referred to as cardiac neural crest cells which gain the ability to migrate along the dorsolateral pathway to the circumpharyngeal region (reviewed by Keyte and Hutson 2012).

Cardiac neural crest cells (CNCC) continue migrating into the pharyngeal arches, with particular localization in the third, fourth, and sixth pharyngeal arches as well as into the cardiac outflow tract (Snider et al. 2007; reviewed by Keyte and Hutson 2012). The cardiac outflow tract temporarily connects the future primitive ventricles with the aortic sac during development of the heart and is where a portion of the cardiac neural crest cells migrate under normal conditions. At murine embryonic day (E) 9.5 the cardiac neural crest cells are migrating into the outflow tract to participate in its septation. CNCCs contribute to the developing heart including forming portions of the septum and contributing muscle and connective tissue to the walls of large arteries (Hutson and Kirby 2003). The aortic arch, ductus arteriosus, and proximal segments of the carotid arteries comprise a significant portion of crest cells as the CNCC associated with the pharyngeal arch arteries contribute to the smooth muscle layer surrounding these vessels (Snider et al. 2007). Cells that migrated within the pharyngeal arches will populate the region and form the pharyngeal arch arteries (PAA). These cells that form the PAA will also participate in the remodeling of these structures. There are five paired arch arteries in mammalian development and they are numbered I to VI. The fifth is believed to be rudimentary, and the first and second PAA mostly disappear during the later stages of embryogenesis with both contributing to other structures (Carlson 2013).

The first will appear in part of the maxillary arteries on both the right and left sides, and the second contributes to the hyoid and stapedial arteries on both sides (Carlson 2013). The PAA remodel with the remodeling of the heart tube as the outflow tract divides into aortic and pulmonary components (Carlson 2013). The third PAA undergoes remodeling and forms the carotid artery system in the developing embryo (Carlson 2013). The fourth and sixth PAAs both undergo asymmetrical remodeling with the development of the heart (Carlson 2013). The left fourth is incorporated into the arch of the aorta, and the right fourth the right subclavian artery. The sixth PAA arises as a capillary plexus and is more appropriately called the pulmonary arch, as it is incorporated into the right and left pulmonary arteries, with the right sixth degenerating and the left sixth forming the ductus arteriosus. (Carlson 2013).

de la Cruz and colleagues showed evidence, as early as 1977, that a second source of myocardium was involved in the complex formation of the heart and that the heart tube formation occurred prior to the addition of the outflow tract (de la Cruz et al. 1977). The first heart field, primitive anterior mesoderm, contributes to the left ventricle, AV canal, and atria and specifically refers to the mesodermal cells that differentiate to form the primordial heart tube. The second or anterior heart field refers to the progenitors added to this heart tube and contribute to the remainder of the heart, including a portion of the right atrium and the base of the outflow tract. While de la Cruz and colleagues were the first to express the thought that there were regions in the heart formed by a second source of myocardium, it was not until 2001 when three different groups molecularly described this population of cells. Using a fibroblast growth factor

(FGF) 10-nlacZ reporter mouse, Kelly and colleagues demonstrated a model that showed expression in the right ventricle and outflow tract myocardium at E9.5, and with labeling techniques, were able to show that this myocardium was added from the pharyngeal arch core and the splanchnic mesoderm (Kelly et al. 2001). Mjaatvedt et al., and Waldo et al. identified sources of the outflow tract myocardium using the embryonic chick with both using cell labeling to determine the origin of these cells (Mjaatvedt et al. 2001, Waldo et al. 2001). These three groups identified and referred to a subpopulation of the secondary heart field based upon their findings, leaving us with slightly varied terminology when referring to this population of cells. The region defined by the FGF 10-lacZ expression is deemed the anterior heart field and consists of the right ventricle and outflow tract progenitors (Kelly et al, 2001). While the secondary heart field identified by Waldo et al., refers to the mesoderm that gives rise to the distal outflow tract, specifically the arterial pole (Waldo et al, 2001). The cardiac neural crest cells play a crucial role in this stage of development as they are needed to induce the migration of these secondary heart field myocytes.

One of the signaling pathways important in cardiac development, including the migration of the CNCC and the rearrangement of the PAAs, involves the function of Endothelin receptor type A (Ednra). There are two Endothelin receptors: Endothelin A receptor and Endothelin B receptor and three associated ligands: Endothelin 1, 2, and 3. Physiological effects of Endothelin B receptor typically oppose the actions of Endothelin A receptor. Activation of Endothelin B receptor occurs normally in conjunction with Endothelin 3. The activation of Endothelin B receptor results in vasodilation, naturiesis

and diuresis as effects to lower blood pressure. Ednrb and Edn3 mutations are associated with Hirschsprung's disease in humans and piebald lethal in horses and inactivation causes similar defects in mice as enteric nerves are not properly formed. The Endothelin A receptor (Ednra) on the other hand is an important signaling molecule known to produce vasoconstrictive effects when it is activated by the ligand Endothelin-1 (Edn1) under normal physiological conditions. Ednra upregulates the production of Edn1 and is the favored receptor for Edn1 and has almost no affinity for Edn2 or 3 (Kedzierski and Yanagisawa 2001). The Endothelin ligand undergoes a process before becoming biologically active. After the furin-like endopeptidases cleave prepro Edn1 into pro Edn1 (formerly big End1), the Endothelin-converting enzyme (ECE) 1 or 2 cleaves pro Edn1. This cleaving process results in the bioactive 21-amino acid peptide known as Edn1 (Kedzierski and Yanagisawa 2001). Both Edn1 and *Ednra* mutants have been shown to present with similar craniofacial and cardiovascular defects (Clouthier et al., 1998; Ruest et al., 2004; Ruest and Clouthier, 2009; Kurihara et al. 2009). Inactivation of the Endothelin converting enzyme genes ECE1 and ECE2 confirms that the Endothelin receptor A / Endothelin 1 pathway has a different function than Endothelin receptor B /Endothelin 3 pathway during development (Yanagisawa et al. 2000).

Ednra is a seven pass transmembrane G protein-coupled receptor. Ednra signaling is involved in several pathological and physiological pathways including vasoconstrictive effects on smooth muscle, patterning, and vessel formation. Ednra is present in the vessels of the kidney, in both the medullar and cortical regions, playing a role in the renin/angiotensin system (Wendel 2006; Neuhofer and Pittrow 2006). Ednra

is associated with aggressive cancers, mainly in bone-localized metastases, and disruptions in the Ednra pathway are linked to inflammatory diseases, such as pulmonary hypertension, as well as other diseases, such as congestive heart failure, cardiac hypertrophy, chronic kidney disease, systemic hypertension, tissue fibrosis and possibly preeclampsia (Zhang, et al. 2013; Barton and Yanagisawa, 2008; George and Granger 2011). Endothelin is also involved in the inflammatory process and may play a role in disease states such as periodontitis (Fujioka, Nakamura, Yoshino et al. 2003). *Ednra* expression is regulated by progesterone, which could potentially explain the association between Ednra and aggressive feminine cancers, such as metastatic breast cancer (Zhang et al. 2013). While the Ednra pathway is known to have implications in pathological and physiological conditions alike, there is much to investigate before this signaling pathway is fully understood (Barton and Yanagisawa, 2008).

*Ednra* is expressed in cranial and cardiac neural crest-derived mesenchymal cells and consequently has an effect on the development of the craniofacial and cardiovascular regions during embryogenesis (Clouthier et al., 1998; Ruest et al., 2004; Ruest and Clouthier, 2009). Mice that are lacking the Endothelin receptor type A suffer from severe craniofacial and cardiovascular defects, causing neonatal lethality (Clouthier et al., 1998; Ruest et al., 2004). Inactivation of *Ednra* signaling results in the homeotic transformation of the mandible into upper jaw-like structures (Ruest and Clouthier, 2009; Ruest et al. 2004). The transformation is caused by the disrupted expression of the homeotic genes *Dlx5* and *Dlx6* in the mandibular pharyngeal arch and inactivation of these two genes causes similar craniofacial defects (Ruest and Clouthier, 2009; Ruest et

al. 2004). Conditional inactivation or the use of a specific receptor antagonist revealed that *Ednra* is one of the earliest mechanisms involved in neural crest cell patterning around E9.0 in the mandibular arch and a similar timing is believed to occur for the posterior pharyngeal arches while these cells are migrating toward the arches and cardiac outflow tract (Ruest and Clouthier 2009).

*Ednra*-/- mice present with defects similar to the cardiovascular defects observed in patients with DiGeorge syndrome. However, the evidence from the analysis comparing the DiGeorge's candidate gene, Tbx1, and Ece1 supports the theory that the Endothelin pathway works independently from the Tbx1/Fgf8 pathway in neural crest-derived structures (Morishima et al., 2003).

The cardiovascular defects seen in *Ednra* mutant embryos commonly include persistent patent ductus arteriosus, coarctation of the aorta, ventricular septal defects and, more rarely, missing or improper attachment of the right subclavian artery, persistent truncus arteriosus and double outlet right ventricle. Potential causes that would explain the cardiovascular defects include migration problems, abnormal apoptosis or proliferation, or problems with patterning. We know the defects occurring in *Ednra* mutant embryos affect the remodeling of the pharyngeal arch arteries and the migration of the CNCC in the cardiac outflow tract (COFT), which occurs at an early stage of development. It has been previously noted that there appears to be inappropriate migration of the cardiac neural crest cells in the outflow tract of *Ednra* mutant embryos (Abe et al. 2007). We introduced *Wnt1-Cre* and *Rosa26R* (R26R) into the *Ednra*-/- line

to determine the fate of the CNCC in the *Ednra*-/- embryos during cardiovascular development.

The result of the fate mapping analysis showed that at E9.5 the CNCC populated normally the caudal pharyngeal arches and arteries in the mutant embryos (Figure 1). Their migration did, however, appear to halt prematurely after entering the truncus portion of the outflow tract. Analysis of E10.5 embryos revealed that the phenotype persisted in the COFT with cardiac neural crest cells located in the proximal portion of the truncus (Figure 2). Fate mapping analysis of E18.5 *Ednra*-/- embryos revealed that cardiac neural crest cells are found in the great arteries but not in the superior portion of the heart (Figure 3). The malformed vascular structures include coarctation of the aorta and patent ductus arteriosus and these structures, though malformed, contain CNCC (Figure 3).

In order to verify the defects are not caused by abnormal apoptosis or proliferation, the TUNEL assay and Brdu labeling was performed to identify apoptotic and proliferating cells. Analysis of the E9.5 embryos did not reveal any significant differences in the proliferation ($p=0.8597$) or survival ($p=0.7111$) percentages of CNCC in the COFT between the wild type and mutant mice (Figure 4). However, significantly fewer CNCC were visible between the endomysium and myomysium in each section ($p<0.0001$) (Fig. 4A). We reanalyzed the apoptotic death at E10.5 since only the apoptosis rate differences were observed in the mandibular arch at that age. No significant differences ($p=0.8325$) were observed between the wild type and mutant embryos (Fig. 4 and data not shown). We concluded that neither apoptosis nor a

reduction in cell proliferation was the cause of the apparent migratory arrest of the CNCC in the COFT.

We hypothesized that the premature arrest of CNCC migration in the outflow tract was likely due to abnormal expression of specific genes that necessary for their migration. In this study, we examined the anatomical expression of genes in an attempt to elucidate the Endothelin signaling pathway and hypothesized that *Ednra* mutants would display altered gene expression when compared to the wildtype embryos. We looked at E9.5 *Ednra* knockout mice when the migratory phenotype appears.
Also, we examined whether the distribution of Rho family of small GTPases was altered in the mutant embryos. These factors, including Rho, Rac and Cdc42, are known for their role in cell migration and possibly in the migration of CNCC in the outflow tract. Our results should help to elucidate the role of Endothelin receptor A signaling during cardiovascular development.

## 2. PROBLEM

Congenital cardiovascular defects affect one in 110 human births. This high rate of defects is a driving force for research in this area, especially involving the etiology of these defects. Elucidating the role of crucial pathways during normal heart development is important in understanding how this process works so we can one day help prevent some of these congenital defects. Even though *Ednra* is known to play a role in many important physiological and pathological pathways, there is much to learn about this crucial signaling pathway during cardiovascular development.

The cardiovascular defects seen in *Ednra* mutant embryos are caused by disrupted gene expression and mainly include persistent ductus arteriosus, coarctation of the aorta and ventricular septal defects, among others. Understanding the changes in gene expression in *Ednra* mutant embryos will aid in understanding the mechanisms involved during the normal and abnormal cardiac development process and may eventually lead to the development of preventative treatments in utero for similar congenital cardiovascular defects.

We know from our fate mapping analysis that the migration pattern of the CNCC in the *Ednra*-/- embryos is aberrant in the outflow tract. The rate of apoptosis and proliferation was normal in the mutant embryos. With the knowledge that the premature arrest of the migration of CNCC in the COFT of *Ednra-/-* embryos was not due to abnormal apoptosis or proliferation, we hypothesized that the premature arrest of cardiac neural crest cells migration pattern was due to improper gene expression during this

stage of development. We began our gene expression analysis in *Ednra* mutant embryos by performing microarray to identify genes whose expression is altered in the mutant embryo and performed whole mount *in situ* hybridization using probes for some of the genes identified. The genes were chosen as they were involved in cell migration, patterning, or had an unknown function and included *Clavesin 1*, *Clavesin 2*, *Netrin G1*, *CCR1*, *Nppa*, *Islet 1*, *Cdc42*, *Zac 1*, and *Bmp 2*. We analyzed the expression in E9.5 murine embryos when the migratory phenotype appears and the CNCC are migrating in the outflow tract region.

# 3. MATERIALS AND METHODS

## *Mice and Genotyping*

The protocol for the use of animals was approved by the Institutional Animal Care and Use Committee at Texas A&M University Baylor College of Dentistry, and the animals used in these experiments were euthanized following the National Institutes of Health guidelines.

*Ednra* mutant embryos were generated and genotyping was done as described previously (Clouthier et al., 1998).

## *RNA Extraction*

RNA was extracted from both embryonic and adult murine tissue using the Trizol method following the manufacturer's instructions (Invitrogen).

## *Reverse Transcripion*

We performed reverse transcription using 5-10 µg of DNase-treated RNA, combined with 5 µl of Hexanucleotide mix and water to reach a final volume of 42 µl. This mixture was placed in a 65°C water bath for 5 minutes and then cooled at room temperature for 10 minutes. To this mixture 10 µl Buffer, 1 µl RNase inhibitor, 5 µl dNTP and 2 µl mmLV Reverse Transcriptase was added and incubated for 1 hour at 37°C.

*Polymerase Chain Reaction*

Polymerase Chain Reaction (PCR) was done using primers specific for *Clavesin 1, Clavesin 2, Netrin G1, CCR1, Nppa, Cdc42, Zac 1, Rgs 5,* and *Bmp 2*. PCR was done again using the initial PCR products as a template and their respective T7 tagged primers. Resulting PCR products were used as a template with the T7 RNA polymerase enzyme to generate the riboprobes for use in whole mount *in situ* hybridization. A 1% agarose gel was used for analyzing PCR products by gel electrophoresis.

*Whole Mount In Situ Hybridization*

Whole mount *in situ* hybridization was done on E9.5 embryos and carried out over a four-day time period. Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Embryos were rinsed with PBT (PBS + 0.1% Tween 20) and placed in 100% methanol then bleached with 6% hydrogen peroxide for 5 hours followed by rinsing in 100% methanol and stored in 100% methanol at -20°C until ready to proceed. Embryos were rinsed in a series of 5 minute methanol/PBT washes (75%, 50%, 25%, PBT only) and treated with approximately 10 μg/ml proteinase K in 2 ml PBT for 7 minutes. Embryos were washed twice for 5 minutes with 2 mg/ml glycine in PBT followed by three 5 minute washes in PBT. Embryos were refixed in fresh 4% paraformaldehyde/ 0.2% glutaraldehyde in PBT for 20 minutes and rinsed twice with PBT. Hybridization involved transferring the embryos to a 1.5 ml eppendorf tube and rinsing with 0.5 ml of hybridization buffer (5X SSC pH 5, 1% SDS, 50 μg/ml yeast

tRNA, 50 µg/ml heparin, 50% formamide), replacing the hybridization buffer with 1ml fresh hybridization buffer after the embryos sunk to the bottom of the tube. Embryos were prehybridized at 65°C for 1 hour with gentle shaking followed by replacing the buffer with 400 µl fresh hybridization buffer with 5 µl probe and were incubated overnight at 65°C with gentle shaking. Washes were performed the following day with two 30 minute washes at 65°C in wash solution 1 (5X SSC, 1% SDS, 50% formamide) followed by a 10 minute wash at 65°C in a 1:1 mix of wash solutions 1 and 2. The embryos were then washed three times for 5 minutes at room temperature in wash solution 2 (0.5 M NaCl, 10mM Tris-HCl pH 7.5, 0.1% tween 20) followed by one wash for 1 hour at 37°C with 100 µg/ml RNase A in wash solution 2. Embryos were washed once for 5 minutes at room temperature in wash solution 2, twice for 30 minutes at 65°C with wash solution 3 (2X SSC, 0.1% tween 20, 50% formamide) and three times with TBST (Tris-buffered saline with 0.1% tween 20). Embryos were pre-blocked with 1 ml of 10% sheep serum in TBST for 1.5 hours which was then removed and replaced with the preabsorbed antibody (3 mg embryo powder to 0.5 ml TBST was heated at 70°C for 30 minutes and cooled on ice. 5 µl of sheep serum and 1 µl of anti-digoxigenin antibody coupled to alkaline phosphatase was added and shaken for 1 hour at 4°C. Solution was centrifuged for 10 minutes at 4°C and the supernatant was recovered and diluted with 1% sheep serum in TBST to a 1:2000 concentration). Embryos were incubated with the preabsorbed antibody overnight at 4°C with gentle shaking. Embryos were rinsed three

times for 10 minutes with TBST followed by transfer to a 15 ml round bottom tube and washed five times for 1 hour each at 4°C with 10 ml of TBST followed by an overnight wash to reduce background. Embryos were washed twice the following morning in fresh NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50mM MgCl2, 0.1% Tween 20) containing 2mM levamisole for 10 minutes and transferred to a glass scintillation vial where they were incubated in 2 ml NTMT (containing 2 mM levamisole with 4.5 µl NBT and 3.5 µl BCIP per ml) in the dark. Embryos were checked every five minutes and when color developed, the embryos were rinsed 5 times with PBT and observed under the stereoscope as described previously.

*Immunohistochemistry*

For the immunohistological assays, E9.5 wildtype and *Ednra*-/- embryos were fixed in 4% paraformaldehyde/PBS, dehydrated in graded ethanols, embedded in paraffin and sectioned at 8 µm along the sagittal plane. After deparaffination and rehydration, the sections were treated either with citric acid or Vector Lab's high pH Antigen Unmasking solution at 90°C for 20 minutes to unmask the antigens. After blocking, the sections were incubated with the recommended concentration of primary antibodies diluted in PBS overnight at 4°C. The next day, the sections were rinsed and incubated with Alexa fluor-coupled secondary antibodies (Invitrogen). The rinsed slides were mounted with Slowfade Gold containing DAPI (Invitrogen) to counterstain the nuclei and then examined by fluorescence microscopy with a Leica TCS SP5 II confocal

microscope fitted for digital imaging. The antibody for Cdc42 was obtained from Abcam, the active RacGTP antibody was purchased from NewEast Biosciences, and Epitomics was the provider of the Rho and Rho GEF antibodies.

# 4. RESULTS

Migration of the cardiac neural crest cells prematurely arrested in the outflow tract of *Ednra* mutant embryos. The phenotype is not due to abnormal apoptosis or cell proliferation. We hypothesized that the aberrant migration pattern of the CNCC and improper rearrangement of the PAA in *Ednra-/-* is caused by altered gene expression. In an attempt to choose the genes most relevant for our study, we performed microarray analysis on E9.5 wildtype and *Ednra* mutant embryos. The heart and pharyngeal arches 3 and 4 were dissected, and RNA was isolated from the area for microarray analysis. From the genes shown to be expressed in the cardiac region at this stage, we selected several genes known to be involved in migration, patterning, cardiac development or with unknown function that we thought would be helpful to identify and potentially elucidate their role in the endothelin signaling pathway during cardiac development.

In order to analyze the genes involved and identify if the selected genes displayed altered expression in *Ednra* mutant embryos, whole mount *in situ* hybridization was done on E9.5 embryos using *Clavesin 1*, *Clavesin 2*, *Netrin G1*, *CCR1*, *Nppa*, *Cdc42*, *Zac 1*, *Islet 1* and *Bmp 2*. All were analyzed using a stereoscope to examine the differences in the wild type and *Ednra* mutant embryos.

*Bmp2*

Bone morphogenetic protein 2 plays a role in the development of bone and cartilage and also cardiac cell differentiation (Ghosh-Choudhury et al. 2003). *Bmp2* is

also involved in the process of epithelial to mesenchymal transition of cells, and activation of key regulatory myocardial genes are dependent on the bone morphogenic protein signaling family, which includes *Bmp2* (Ghosh-Choudhury et al. 2003; Buckingham, Meilhac, Zaffran, 2005). This factor is important in early embryogenesis for mesoderm formation and cardiac development. Knockout of *Bmp2* results in embryonic lethality while *Bmp2* deficient mice display cardiac defects (Wang et al. 2014). We observed that the *Ednra* -/- had increased *Bmp2* expression in the ventricle and COFT when compared to the wildtype (Figure 5). These results match a previous publication indicating that endothelin signaling was needed to repress cardiac Bmp2 expression during cardiac development (Asai et al 2010).

*Zac1*

Zac1 is a zinc finger transcription factor involved in regulating apoptosis and cell cycle arrest and is known to play a role in cardiovascular development. Knockout of Zac1 results in cardiovascular defects including atrial septal defects as well as ventricular septal defects (Yuasa et al., 2010; Varrault et al. 2006). Zac1 is expressed in the heart during its cardiac crescent stage and is important for morphogenesis (Yuasa et al, 2010). The Zac1 knockout also presents with fetal growth restriction (Varrault et al. 2006). Zac1 has also been identified by Yuasa et al. as a potential activator of Nppa gene expression (Yuasa et al. 2010). The *Ednra* mutant embryos displayed increased expression of Zac1 in the bulbus cordis and left ventricle. Expression also appeared to be slightly increased in the cardiac outflow tract of the *Ednra* mutant. The *Ednra* mutant

also presents with higher Zac1 expression in the crest cells around the arch arteries when compared to the wildtype embryo (Figure 5).

*CCR1*

Chemokine receptor 1 (*CCR1*), also designated CD191 (cluster of differentiation 191), is important for wound healing, recruitment of immune cells as well as neutrophil trafficking and proliferation (Khan et al. 2001). *CCR1* is involved in embryologic development and angiogenesis (Pease and Horuk 2009; Mortier et al. 2008). In the E9.5 *Ednra* mutant, *CCR1* expression was lower in the bulbus cordis, future left ventricle, and in crest cells mainly around where the cranial nerves develop. Upon dissection, it also appeared that the *Ednra-/-* presents with lower *CCR1* expression in the cardiac outflow tract when compared to the wildtype (Figure 5).

*Nppa*

Natriuretic peptide A (Nppa), also called atrial natriuretic peptide (ANP), is an important modulator of cardiac hypertrophy and knockouts suffer from exaggerated cardiac enlargement, left ventricular hypertrophy and increased blood pressure (Mori et al. 2004). Because *Nppa* is associated with maturation of the left ventricle, we expected to see expression changes during cardiac development (Buckingham, Meilhac, Zaffran, 2005). Figure 5 shows expression of *Nppa* during this stage of development and the *Ednra* mutant presents with weaker *Nppa* expression in the cardiac outflow tract when compared to the wildtype but ventricular expression did not appear to be altered.

*Netrin G1*

*Netrin G1*, also called laminet-1, is in the Netrin family and normally found in the nervous system, pancreas, and cardiac muscle tissue. The Netrin family is known to guide cell migration and the primary role of Netrin G1 is in axonal guidance. Knockout of Netrin G1 results in selective mislocation of receptors but not axonal mistargeting (Barallobre et al. 2005; Nishimura-Akiyoshi et al. 2007). *Netrin G1* is associated with neural crest differentiation as well as providing signals for synapse development (Matsukawa et al., 2014; Cavard et al. 2009). The *Ednra* -/- displayed a stronger expression of *Netrin G1* in the cardiac outflow tract and ventricle than the wild type at E9.5 (Figure 5).

*Clavesin 1*

*Clavesin 1 (CLVS1)* is sometimes referred to as retinaldehyde binding protein 1 and, while its normal function is not fully understood, it is probably a nucleolar protein or a vesicle protein. In 2009, Katoh and colleagues performed specific knockdowns of the clathrin vesicle associated Sec14 protein in neurons and discovered it does play a role in neuronal function, however, *Clavesin 1* function is still greatly unknown (Katoh et al. 2009). Analysis of the gene expression in E9.5 embryos showed no significant difference in *Clavesin 1* between the *Ednra* -/- and the wildtype mice in neural crest cells (Figure 6). E9.5 wildtype and *Ednra* -/- have grossly similar *Clavesin 1* gene expression; however, upon dissection of the heart, it became apparent that the expression in the

bulbus cordis, the future right ventricle of the *Ednra* mutant, was significantly weaker than in the wildtype (Figure 6). The ventricles and arches in the wildtype and *Ednra* mutant hearts did not appear to have any significant differences in *Clavesin 1* expression (Figure 6). Analysis of the anatomical expression of *Clavesin 1* in E10.5 embryos shows obvious expression in what appears to be all crest cells in the wild type whereas the *Ednra* mutant shows almost complete lack of expression throughout the entire embryo. The E10.5 *Ednra* mutant did display nominal expression on the limbs, but was otherwise lacking in *Clavesin 1* gene expression.

*Clavesin 2*

*Clavesin 2* is also a clathrin vesicle associated Sec14 protein whose function is largely unknown, however, it is believed to be important in normal morphology of endosomes and lysosomes in neurons (Katoh et al. 2009). *Clavesin 2* was selected due to its similarity to *Clavesin 1*. Since the normal function of this gene is unclear, we were unsure what the results of our analysis may show. Upon observation of the wild type and *Ednra* mutant embryos, no significantly apparent differences in *Clavesin 2* gene expression were noted between the two genotypes (Figure 6).

*Islet 1*

*Islet 1* is responsible for encoding the insulin gene enhancer protein ISL1. *Islet 1* known to play a role in secondary heart field development and is considered a marker for cardiac progenitors of the secondary heart field though it also labels cardiac neural crest

cells (Asai et al., 2010; Engleka et al. 2012). Mutations in the *Islet 1* gene result in complete lack of the outflow tract (Buckingham, Meilhac, Zaffran, 2005). We did see the expression of *Islet 1,* however, we did not observe any difference in *Islet 1* gene expression between the wildtype and *Ednra*-/- embryos (Results not shown). This indicates the migration of the second heart field myocytes is occurring normally in the absence of Endothelin receptor A, and the defects are due to migration of the CNCC rather than issues with the second heart field myocytes.

*MF20*

To confirm that the migration defect is affecting the CNCC and not the second heart field myocytes, we looked for the presence of myocytes with the MF20 antibody at E10.5. The analysis confirmed what we saw initially with the expression of Islet 1 and we saw no difference in expression in the wildtype and mutant embryos (results not shown).

*Rho Family of GTPases*

Upon completing our anatomical analysis of gene expression in *Ednra* mutant embryos, we have identified a number of genes that display altered expression in mutant embryos that could potentially explain the heart defects seen in these mutants. However, with identifying the anatomical expression of these genes, we could not explain the CNCC migration phenotype in the cardiac outflow tract. We decided to analyze the Rho family of small GTPases known for their role in cell migration. The Rho family of

GTPases are involved in the regulation of many aspects of intracellular actin dynamics and play a role in organelle development, cytoskeletal dynamics and, notably, cellular movement.

We examined E9.5 wild type and *Ednra* mutant embryos to determine the presence of small GTP-binding proteins Rho and Rho GEF, Rac1 and 2, and Cdc42. We saw no difference between the wildtype and mutant when looking at the immunohistochemistry for Rho and Rho GEF (results not shown). Our analysis of the location of Rac GTP proteins showed no differences in the Rac GTP proteins, the activated form of Rac 1 and 2, in the cardiac neural crest cells (Figure 7). However, there appeared to be greatly increased expression of Rac GTP in the myomesium of the *Ednra* mutant (Figure 7).

We performed immunohistochemistry using Cdc42 and observed abundant localization in all cells in the wildtype with brightly visible signal in the NCC migrating in the COFT. In the mutant, Cdc42 activation in the NCC of the cardiac outflow tract was not observed but was normally present in the other tissues. Immunohistochemistry revealed that Cdc42 failed to localize at the migratory fronts in the CNCC in the *Ednra* mutant embryos (Figure 7). We also performed whole mount *in situ* hybridization to observe Cdc42 expression in the heart. Expression was great throughout the heart and we were not able to identify any differences in Cdc42 expression between the wildtype and the mutant due to the high level of expression in this area at this stage of development.

With RacGTP expression abnormal in the myomesium of the mutant embryos and Cdc42 abnormal in the CNCC of *Ednra* mutants, we can conclude there are issues with the localizing proteins that contribute to the premature arrest of the CNCC in the outflow tract of these mutants.

# 5. SUMMARY AND CONCLUSION

Normal *Ednra* function is crucial for proper development. Endothelin signaling is involved in neural crest cell migration, and inactivation of the gene encoding the Endothelin A receptor in the murine model results in characteristic defects including craniofacial and cardiovascular defects. The absence of Ednra signaling in cephalic NCC results in the transformation of the mandible into maxillae-like structures (Ruest and Clouthier, 2009; Ruest et al., 2004).

The cardiovascular defects seen in *Ednra* mutant embryos include persistent patent ductus arteriosus, coarctation of the aorta and ventricular septal defects. Ednra is perhaps one of the earliest mechanisms involved in neural crest cell patterning around E9.5 and earlier while these cells are migrating toward the arches and cardiac outflow tract (Ruest and Clouthier 2009). The defects seen in *Ednra* mutant embryos are caused by disrupted gene expression and further elucidation of the Ednra signaling pathway, along with identifying the key players involved in this signaling pathway, is essential to understanding this complex signaling pathway and its role in development.

Fate mapping analysis revealed the migratory pattern of the cardiac neural crest cells in the pharyngeal arches and arteries is normal in the *Ednra* mutant embryos and that these cells arrest prematurely prior to populating the cardiac outflow tract. The migration is aberrant upon reaching the truncus portion of the outflow tract at E9.5 in the E18.5 mutant embryos, the CNCC appear to normally populate the great arteries but fail to populate the superior portion of the heart. *Ednra* mutants display malformed vascular

structures, but these structures do contain CNCC showing that the migration of the CNCC occurs normally until they reach the OFT in mutant embryos.

Cell viability and proliferation appeared normal in E9.5 mutant embryos. Asai and colleagues previously stated that they did not see a difference in the proportion of apoptotic cells in the COFT when comparing *Ednra* mutants to both the wildtypes and heterozygotes (Asai et al. 2010). We expected to see similar results and upon our confirming that the premature arrest of CNCC was not due to increased apoptosis or decreased proliferation, it became evident that the arrest of the cardiac neural crest cells during their population of the outflow tract is likely due to the failure of the activation of expression of specific genes necessary for the migration of these CNCC.

The over expression of *Netrin G1* appears to occur only in the COFT of the *Ednra* mutant embryos, while the over expression of *Zac1* occurs in the mutant left ventricle and bulbus cordis as well as in crest cells around the arches.

The expression of *Nppa* was weaker in the cardiac outflow tract of *Ednra* mutant embryos, and *CCR1* was also weaker in the outflow tract, bulbus cordis and in the crest cells around where the cranial nerves develop in *Ednra* mutant embryos.

*Clavesin 1* gene expression in E9.5 embryos show grossly similar expression between the wildtype and mutant but upon dissection, it was apparent the expression in the bulbus cordis of the *Ednra* mutant was significantly weaker than that of the wildtype. We chose to further analyze the expression of this gene at E10.5 and the *Clavesin 1* expression was prevalent in what appear to be all crest cells in the wildtype, with near complete lack of expression with nominal expression on the limbs in the *Ednra* mutant.

These findings are significant in that we have identified that *Clavesin 1* gene expression is regulated early on by the *Ednra* signaling pathway and is essential in the coordination of the migration of cardiac neural crest cells in the outflow tract. While the function of *Clavesin 1* is not yet understood, one possible function for *Clavesin 1* is as a retinaldehyde binding protein. The neural crest cells populating the conotruncal ridges appear to be affected by the knockout of the retinaldehyde receptors (Ghyselinek et al. 1998). The link between the knockout of the Endothelin receptor A and the retinaldehyde receptors leads us to presume that they are potentially involved in the same pathway. Coupling the results we have shown with the change in *Clavesin 1* gene expression in *Ednra* mutants and the belief that *Clavesin 1* is a retinaldehyde binding protein, we can presumably link the activities of retnoic acid and the *Ednra* pathway.

Previous studies have shown at E8.5 that there is no obvious change of *Bmp2* expression in *Ednra* mutant embryos and at E9.5 the expression is increased (Asai et al., 2010). We saw increased expression in the left ventricle as well as in the pharyngeal arch arteries and the aortic sac. These results are consistent with previous findings of increased *Bmp2* gene expression in the heart of *Ednra*-/- embryos. BMP signaling has been known to play a role in second heart field development. Prall et al. showed, using a Nkx2.5 null mouse, that the BMP signaling pathway is important in holding the second heart field in a proliferative state as the first heart field differentiates (Prall et al. 2007). This process occurs with Nkx2.5 normally acting as a Smad1 inhibitor which results in downregulation of *Bmp2* signaling and holds the second heart field progenitor cells inactive during the development of the initial heart tube (Prall et al. 2007). Because the

inhibition of *Bmp2* signaling is involved in holding the second heart field in a proliferative state, we expected the signaling to be active during the differentiation of the second heart field progenitors. The study done by Prall et al. displayed this clearly by examining a Nkx2.5 null mouse in which the expression of BMP was not inhibited, thus there were no progenitors to add during second heart field differentiation as they had already differentiated with the first heart field (Prall et al., 2007). The increased *Bmp2* expression in the *Ednra* mutant embryos in the left ventricle, pharyngeal arch arteries, and aortic sac, suggest that knockout of the Ednra pathway results in uncontrolled *Bmp2* expression, however, the consequences of increased expression are unknown.

*Islet 1* did not show any difference in expression between the *Ednra* mutant and the wildtype. This result was expected as the expression of *Islet 1* is seen in nearly the entire progenitor population of heart cells and the expression is expected to diminish during myocardial differentiation (Sun et al., 2007). *Islet 1* has previously been identified as a heart field marker, and lineage analysis has traced these cells to the right ventricle, outflow tract, atria and portions of the left ventricle (Yuan and Schoenwolf, 2000; Cai et al. 2003). Knockout of *Islet 1* supports these lineage tracings as the knockout lacks these regions (Cai et al. 2003). The crest cells, on the other hand, are involved in both the formation of the COFT and rearrangement of the pharyngeal arch arteries. Because we did not see any difference in gene expression of *Islet 1* in the *Ednra*-/- we can conclude that the migration of the second heart field myocytes is occurring normally and that the migratory phenotype solely affects the cardiac neural crest cells.

To confirm this finding, we looked for the presence of myocytes with the MF20 antibody at E10.5 and saw no difference between the wildtype and mutant at this stage. This confirms that the migratory phenotype affects the cardiac neural crest cells and not the second heart field myocytes.

After identifying several genes and confirming our hypothesis that there is altered gene expression in *Ednra* mutant embryos, we decided to look a step further and try to answer why these CNCC stop their migration in the COFT of *Ednra* mutants. To answer this question, we looked into the presence of small GTPase localizing factors. One small GTPase involved in the regulation of cellular activities, including cell migration as well as establishing cell polarity, is Cdc42 which cycles between GDP-bound and GTP-bound switching between the inactive and active state (Erickson and Cerione, 2001; Etienne-Manneville and Hall, 2002; Cerione, 2004; Etienne-Manneville, 2004; Heasman and Ridley, 2008; Raftopoulou and Hall, 2004). Cdc42 is in the Rho family and conditional knockout of Cdc42 in crest cells has shown to result in craniofacial and cardiovascular defects similar to the *Ednra* knockouts including persistent truncus arteriosus, hypoplastic pulmonary trunk, outflow tract defects and abnormal aortic arch artery patterning (Liu et al. 2013).

We analyzed the location of Rac 1 and 2 as well as Rho GCF and no differences were observed in the Rac GTP proteins or the Rho GCF in the CNCC, however, the Rac GTP proteins appeared to be increased in the myomesium of the Ednra mutant embryos likely indicating an issue with the signaling factors involved in this process. Our data also revealed that Cdc42 fails to localize properly in the migratory fronts of the CNCC

of mutant embryos. We can assume that the migratory defect seen in the *Ednra* mutant embryos is related to the disruption of Cdc42 activity in the CNCC migrating in the COFT and is also related to the improper RacGTP activity in the myomesium. Our data shows that Cdc42 activation appears to be in CNCC migrating in the COFT but not in the CNCC within the pharyngeal arches suggesting an important function for the protein in CNCC migration along the COFT. Our data also reveal that Cdc42 activation via Ednra signaling occurs early, and these results support the idea of the function of Cdc42 as a downstream effector of Ednra signaling in the CNCC migrating in the COFT. Improper rearrangement of the pharyngeal arch arteries, as seen in the *Ednra* knockout as well as noted by Liu and colleagues in their Cdc42 conditional knockout, suggests that Cdc42 may also participate in the patterning and remodeling of the arteries.

While we are continuing to elucidate the complex Ednra pathway in hopes of one day preventing the high prevalence of cardiovascular defects, much research still needs to be done in this area. In the future, we hope to further identify genes involved in this pathway, as well as identify downstream targets of these genes. While we have identified several genes that display altered expression and have also shown a link between Cdc42 and the Ednra pathway, we hope to investigate the migration pattern further by perhaps performing laser capture in the future for microarray and analysis of more localized gene expression to further identify why the CNCC stop migrating when they reach the OFT.

In conclusion, loss of the Endothelin receptor A signaling results in severe cardiovascular defects. However, these defects result mostly from improper patterning of cardiac neural crest cells during pharyngeal arch artery rearrangement. Cardiac defects

result from small gene expression changes in the developing heart and the Endothelin dependent patterning genes regulate the rearrangement of the pharyngeal arch arteries. Migratory defects of CNCC do not affect myocyte development from the secondary heart field. Also, it appears that there may be two subpopulations of CNCC : one independent and one dependent on the Endothelin receptor A signaling pathway for their migration. Finally, in the cardiac outflow tract neural crest cells, Endothelin receptor A signaling involves Cdc42 activity.

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APPENDIX A

FIGURES

**Figure 1**

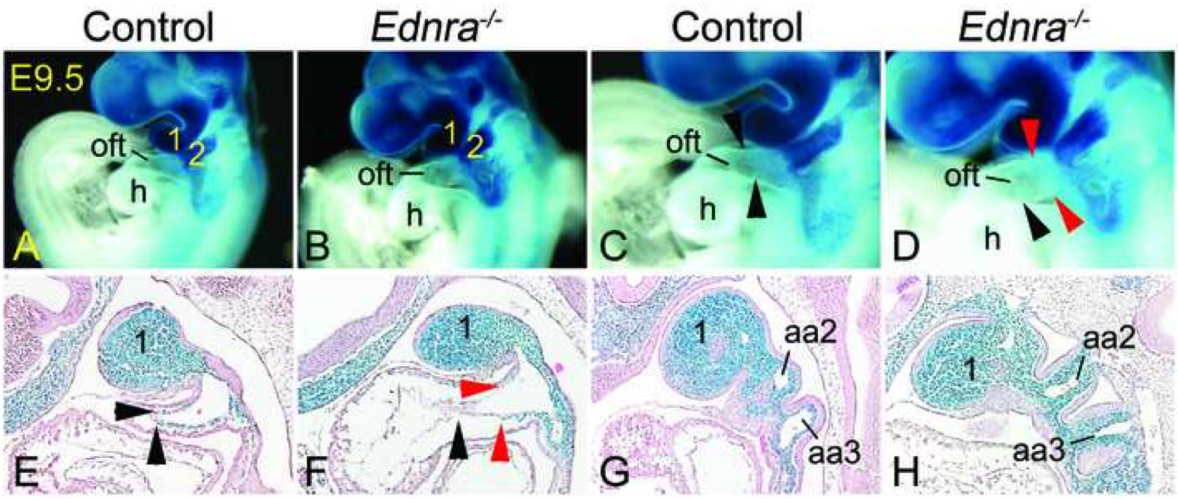


**Fig. 1.** Analysis of cardiac neural crest cell migration in E9.5 *Ednra*-/- embryos. A, B. Lateral views of whole-stained embryos carrying the R26R and Wnt1-Cre alleles revealing the location of CNCC, stained in blue, in the pharyngeal arches 1 and 2 and COFT (oft) in control and Ednra- /- littermates. C, D. Enlarged views of the same embryos. The black arrowheads in C indicate the location of the CNCC migration front in the COFT. The same location was reported in the mutant embryo in D but the actual CNC migration front is indicated by the red arrowheads in this embryo. E-H. Sagittal sections from the whole-stained embryos and counterstained with eosin. The black arrowheads indicate the location of the CNCC in the wild type COFT and the relative position in the mutant embryo (E and F) while their actual location in the Ednra-/- embryo is indicated by the red arrowhead. CNCC are normally present in the pharyngeal arches and around the pharyngeal arch arteries (aa) 2 and 3 in the mutant embryo. h, heart.

**Figure 2**

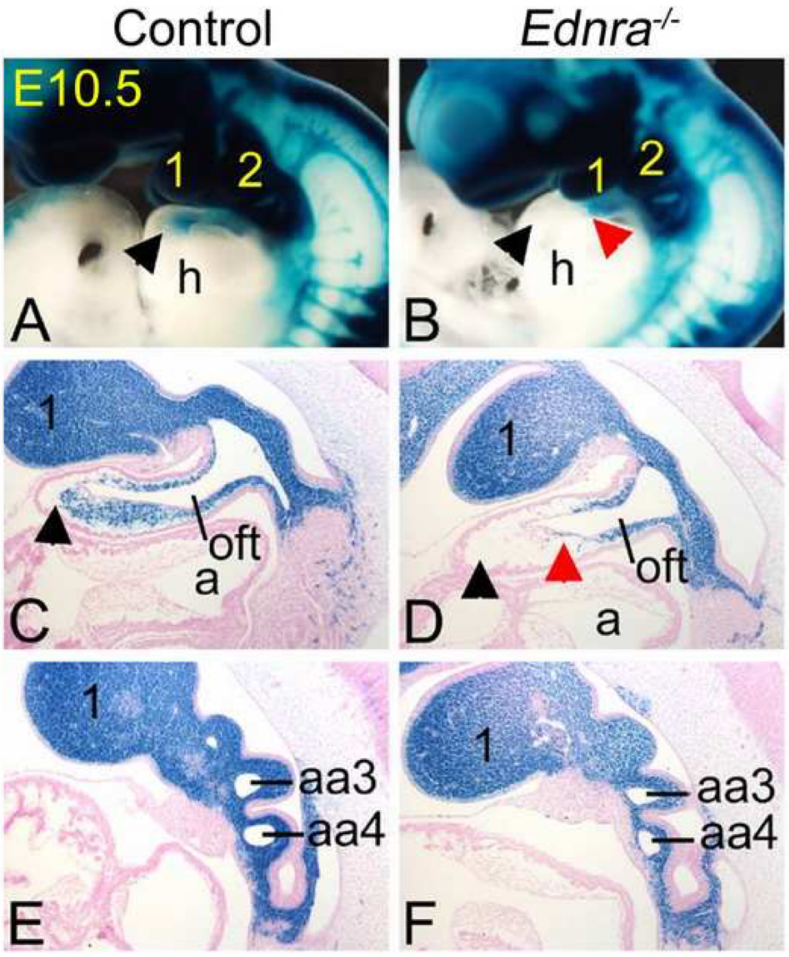


**Fig. 2.** Analysis of CNCC distribution in whole-stained E10.5 *Ednra*-/- embryos. A, B. Lateral views of whole-stained embryos. The black arrowheads indicate the location or relative location of wild type CNCC and the red arrowhead indicates the actual location of the CNCC in the mutant embryo. C-F. Sagittal sections, counterstained with eosin, through the COFT (oft) and pharyngeal arch arteries (aa) 3 and 4. 1, mandibular arch; 2 pharyngeal arch 2; a, atria; h, heart.

**Figure 3**

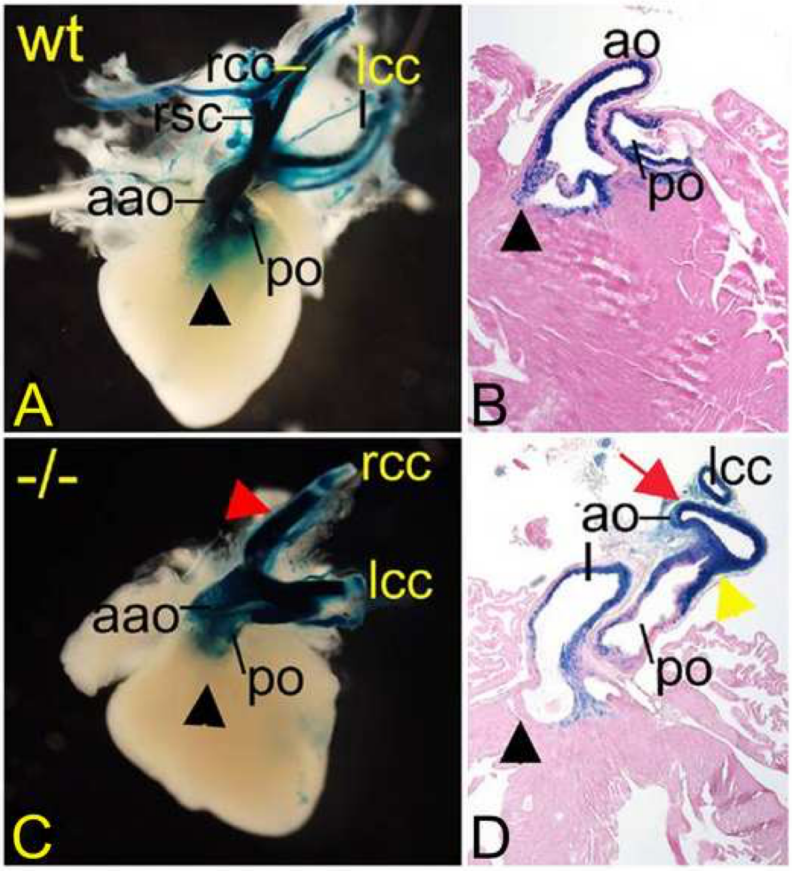


**Fig. 3.** Analysis of CNCC distribution in whole-stained E18.5 *Ednra*-/- hearts. Sections were counterstained with eosin. The black arrow in A indicates the location of the CNCC in the wild type (wt) heart and the equivalent location in the mutant (-/-) heart in C. The red arrowhead in C points to the location where the right subclavian artery (rsc) is missing. The black arrowhead on the section in B points to the presence of CNCC at the base of the ascending aorta (aao) but their absence in D in the mutant heart. The yellow arrow in D indicates the presence of CNCC in the patent ductus arteriosus at the connection site with the aorta (red arrow). ao, aorta; lcc, left common carotid; po, pulmonary outflow; rcc, right common carotid.

**Figure 4**

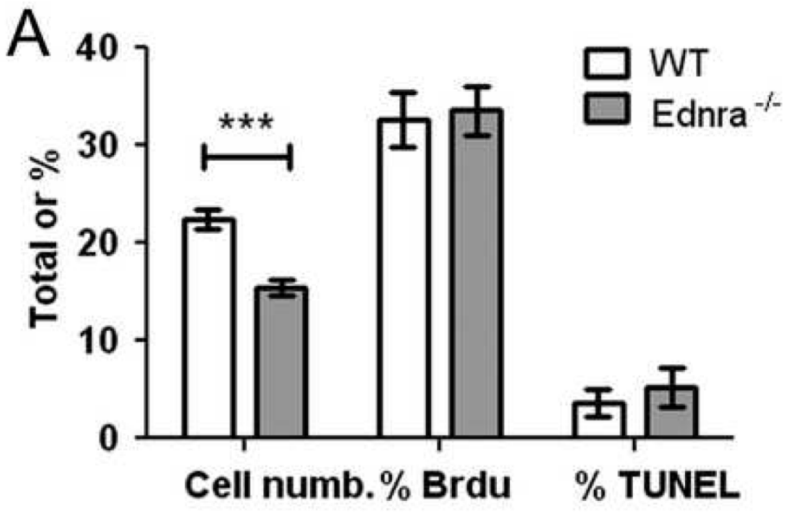


**Fig. 4.** Analysis of CNCC proliferation and survival in E9.5 and E10.5 *Ednra*-/- embryos. A. Total CNCC numbers or percentages of proliferating and apoptotic cells in the COFT of E9.5 wild type (WT) and Ednra-/- embryos. ***, $p<0.005$

**Figure 5**

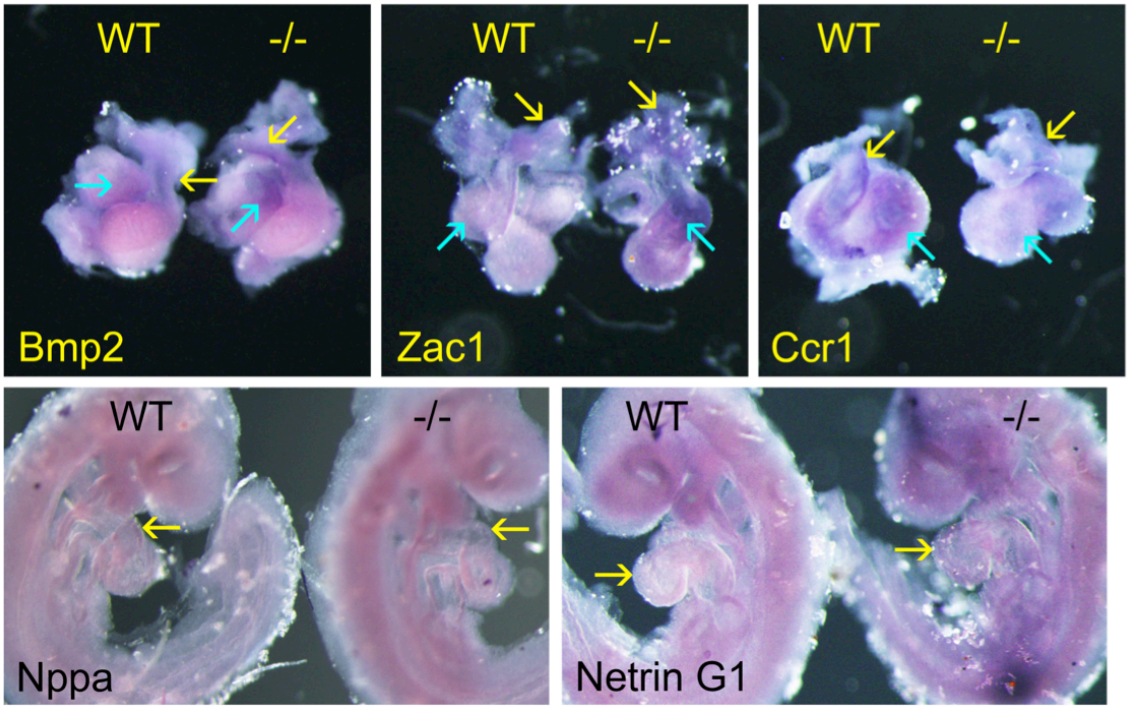


**Fig. 5.** Analysis of gene expression in E9.5 *Ednra* mutant embryos. Bmp2: the mutant presented with increased expression in the ventricle and the outflow tract. Zac1: the mutant presented with increased expression in the bulbus cordis and arch arteries. Ccr1: there was a much smaller and deformed mutant heart which displayed decreased expression in the bulbus cordis, the future right ventricle, and the outflow tract. Nppa: whole embryo shows decreased expression in the outflow tract area of the mutant. Netrin G1: the mutant presents with increased expression in the outflow tract and ventricle.

**Figure 6**

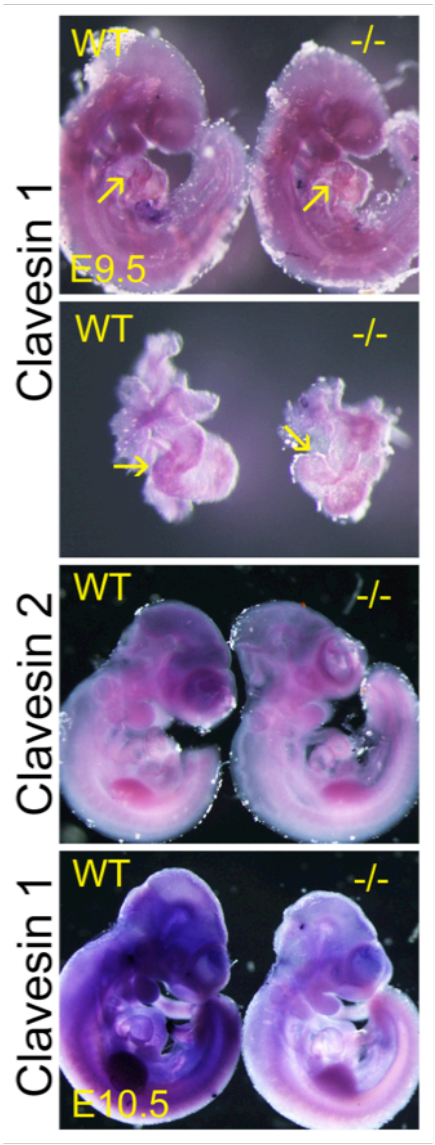


**Fig. 6**. Analysis of gene expression in E9.5 and E10.5 embryos. Clavesin 1: top box shows the whole embryo with a slight decrease in expression in the cardiovascular region. The second box shows the dissected heart and arrows indicate the decrease in expression in the mutant bulbus cordis. Clavesin 2: whole embryos show no difference in expression at E10.5. Clavesin 1: E10.5 whole embryos reveal expression in what appears to be all crest cells in the wildtype with almost complete lack of expression in the mutant with nominal expression in the limbs.

**Figure 7**

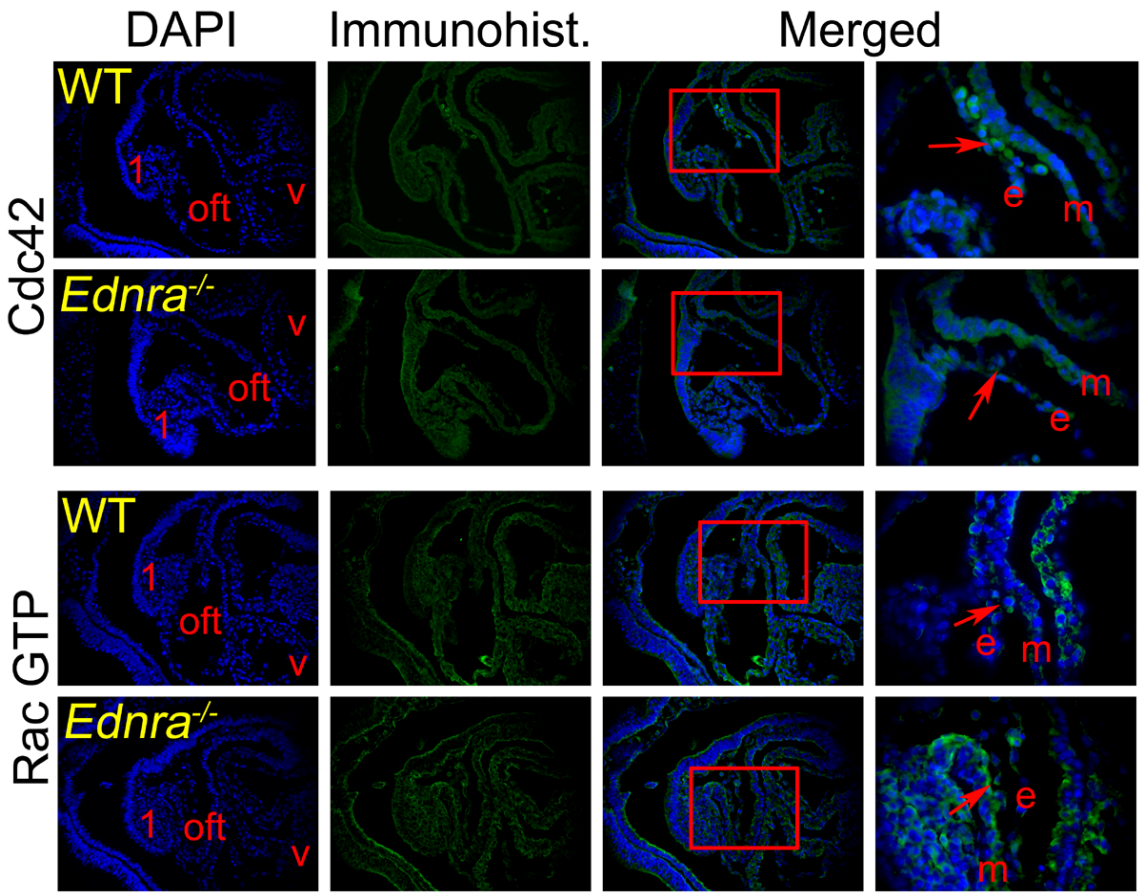


**Fig. 7.** Cdc42 fails to localize properly in the CNCC of *Ednra*-/- embryos. Sagittal sections through the cardiac region of E9.5 wild type (WT) and Ednra-/- embryos were counterstained with DAPI to reveal the nuclei. We examine the presence and localization of the small GTP binding proteins Cdc42 and Rac1 and 2 (activated form, Rac GTP) in the CNCC (red arrows in the enlarged view from preceding photograph) migrating in the outflow tract (oft). No differences were observed for the localization of the Rac GTP proteins. However, in the mutant embryos, Cdc42 fails to localize at the edges of migrating CNCC, as revealed in the enlarged merged images. The enlarged area is framed by a red box. 1, pharyngeal (mandibular) arch 1; e, endomysium; m, myomysium; v, ventricle.